

research. Use for software development or design or implementation of commercial gateways or other similar uses is prohibited and may result in loss of user privileges and other penalties.

* * * * * STN Columbus * * * * *

FILE 'HOME' ENTERED AT 11:39:30 ON 26 JAN 2005

=> file agricola biosis embase caplus

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	0.21	0.21

FILE 'AGRICOLA' ENTERED AT 11:39:43 ON 26 JAN 2005

FILE 'BIOSIS' ENTERED AT 11:39:43 ON 26 JAN 2005

Copyright (c) 2005 The Thomson Corporation.

FILE 'EMBASE' ENTERED AT 11:39:43 ON 26 JAN 2005

COPYRIGHT (C) 2005 Elsevier Inc. All rights reserved.

FILE 'CAPLUS' ENTERED AT 11:39:43 ON 26 JAN 2005

USE IS SUBJECT TO THE TERMS OF YOUR STN CUSTOMER AGREEMENT.

PLEASE SEE "HELP USAGETERMS" FOR DETAILS.

COPYRIGHT (C) 2005 AMERICAN CHEMICAL SOCIETY (ACS)

=> s maize and immature(w)embryo and transform?

L1 116 MAIZE AND IMMATURE(W) EMBRYO AND TRANSFORM?

=> duplicate remove l1

DUPLICATE PREFERENCE IS 'AGRICOLA, BIOSIS, EMBASE, CAPLUS'

KEEP DUPLICATES FROM MORE THAN ONE FILE? Y/(N):n

PROCESSING COMPLETED FOR L1

L2 63 DUPLICATE REMOVE L1 (53 DUPLICATES REMOVED)

=> s l2 yr <2000

MISSING OPERATOR L2 YR

The search profile that was entered contains terms or nested terms that are not separated by a logical operator.

=> d l2 30 ibib

L2 ANSWER 30 OF 63 CAPLUS COPYRIGHT 2005 ACS on STN

ACCESSION NUMBER: 1999:276186 CAPLUS

DOCUMENT NUMBER: 131:126061

TITLE: Establishment of a genetic ***transformation*** system for ***maize*** inbred P9-10

AUTHOR(S): Zhou, Fengyong; Wang, Guoying; Xie, Youju; Cui, Hongzhi; Guo, Sandui; Dai, Jingrui

CORPORATE SOURCE: College of Plant Science and Technology, China Agricultural University, Beijing, 100094, Peop. Rep. China

SOURCE: Chinese Science Bulletin (1999), 44(7), 624-627

CODEN: CSBUEF; ISSN: 1001-6538

PUBLISHER: Science in China Press

DOCUMENT TYPE: Journal

ANSWER 28 OF 63 BIOSIS COPYRIGHT (c) 2005 The Thomson Corporation. on
STN

ACCESSION NUMBER: 2000:281888 BIOSIS
DOCUMENT NUMBER: PREV200000281888
TITLE: Stable **transformation** of plant cells.
AUTHOR(S): Tomes, Dwight T. [Inventor, Reprint author]; Weissinger,
Arthur [Inventor]; Sanford, John C. [Inventor]; Klein,
Theodore M. [Inventor]
CORPORATE SOURCE: Cumming, IA, USA
ASSIGNEE: Pioneer Hi-Bred International, Inc., Johnston,
IA, USA
PATENT INFORMATION: US 5990387 November 23, 1999
SOURCE: Official Gazette of the United States Patent and Trademark
Office Patents, (Nov. 23, 1999) Vol. 1228, No. 4. e-file.
CODEN: OGUPE7. ISSN: 0098-1133.
DOCUMENT TYPE: Patent
LANGUAGE: English
ENTRY DATE: Entered STN: 6 Jul 2000
Last Updated on STN: 7 Jan 2002

AB The invention provides methods for producing a fertile, stably
transformed, Zea mays plant. The methods comprise the steps of
(a) providing a foreign DNA comprising an expression vector carrying a
gene encoding an agronomic trait; (b) providing a **maize**
embryogenic callus, suspension culture, or **immature**
embryo isolated from a plant; (c) introducing the foreign DNA into
the embryogenic callus, suspension culture or **immature**
embryo isolated from a plant by one or more microparticle
bombardments; and (d) regenerating fertile transgenic Zea mays plant.

L3 ANSWER 33 OF 63 CAPLUS COPYRIGHT 2005 ACS on STN

ACCESSION NUMBER: 1999:502597 CAPLUS
DOCUMENT NUMBER: 131:347100
TITLE: Transgenic cereals - Zea mays (**maize**)
AUTHOR(S): Gordon-Kamm, W. J.; Baszczyński, C. L.; Bruce, W. B.;
Tomes, D. T.
CORPORATE SOURCE: Pioneer Hi-Bred International, Inc., Johnston, IA,
50131, USA
SOURCE: Advances in Cellular and Molecular Biology of Plants
(1999), 5 (Molecular Improvement of Cereal Crops),
189-253
CODEN: ACMBEF; ISSN: 1381-1932
PUBLISHER: Kluwer Academic Publishers
DOCUMENT TYPE: Journal; General Review
LANGUAGE: English

AB A review with many refs. Genetic **transformation** of
maize is routine in several genotypes despite the many
difficulties encountered in developing reliable **transformation**
techniques in this major cereal species. Aspects of **maize**
tissue culture, including the target explant, subsequent rapid in vitro
proliferation and dependable regeneration from competent cells were
prerequisite developments for gene delivery into **maize**.
Recovery of transgenic, fertile **maize** required high levels of
gene expression and identification of new selectable markers, along with
DNA delivery into competent **maize** cells. DNA delivery by
particle bombardment, Agrobacterium, electroporation and silica fiber
methods have been the most carefully documented, each of which can now be
used for gene transfer into **maize**. Promoters such as those from
the CaMV 35 S or ubiquitin genes, together with various introns have been
widely used to achieve high expression levels, while the herbicide
resistance gene, bar, has served as an important selectable marker for
numerous studies in **maize transformation**. Although
tissue culture cells were instrumental in the development of **maize**
transformation, the direct use of explants such as the
immature embryo and/or meristems has found favor in more

recent applications. Gene delivery in **maize** has shifted from emphasis on technol. development to evaluation of gene expression with various transgenes, some of which are already in large-scale com. development (e.g. insect and herbicide resistance). **Maize transformation** is increasingly being used to address more sophisticated aspects of gene regulation, plant development and physiol. The stability of transgene expression in primary transgenic plants and subsequent generations is of obvious academic and com. importance. The isolation of promoters with a variety of expression profiles that are tissue-specific and/or temporally regulated will become more important as trait modification strategies evolve. Technologies such as site-directed integration, homologous recombination, 'chimeraplasty', and others will likely become routine in higher plants such as **maize** as this research area, now in its infancy, continues to develop. These technologies have the potential to aid our understanding of gene regulation, and to more directly make changes in endogenous gene sequences or to permit targeting of new genes (or regulatory elements) into precise genomic locations. With an assortment of accompanying genetic tools such as reverse genetic methods, mapping, genome-scale anal. and gene expression information, **maize transformation** has evolved into an important tool for both basic and applied studies in plants.

REFERENCE COUNT: 325 THERE ARE 325 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L3 ANSWER 43 OF 63 CAPLUS COPYRIGHT 2005 ACS on STN

ACCESSION NUMBER: 1996:295123 CAPLUS

DOCUMENT NUMBER: 124:309571

TITLE: Preparation of stably **transformed** transgenic cereal plants by microparticle bombardment of meristematic tissue from **immature embryos**

INVENTOR(S): Bowen, Benjamin A.; Lowe, Keith; Ross, Margot C.; Sandahl, Gary A.; Tomes, Dwight T.; Songstad, David D.; Gordon-kamm, William J.

PATENT ASSIGNEE(S): Pioneer Hi-Bred International, Inc., USA

SOURCE: PCT Int. Appl., 50 pp.

CODEN: PIXXD2

DOCUMENT TYPE: Patent

LANGUAGE: English

FAMILY ACC. NUM. COUNT: 1

PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
-----	---	-----	-----	-----
WO 9604392	A2	19960215	WO 1995-US8977	19950726
WO 9604392	A3	19960328		
W: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT				
RW: KE, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
US 5736369	A	19980407	US 1995-483091	19950607
CA 2195206	AA	19960215	CA 1995-2195206	19950726
AU 9536247	A1	19960304	AU 1995-36247	19950726
AU 697373	B2	19981001		
EP 772687	A2	19970514	EP 1995-933706	19950726
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, LU, MC, NL, PT, SE				
BR 9508341	A	19970812	BR 1995-8341	19950726
HU 76841	A2	19971128	HU 1997-247	19950726
JP 10503374	T2	19980331	JP 1995-506538	19950726

ZA 9506302	A	19960314	ZA 1995-6302	19950728
PRIORITY APPLN. INFO.:			US 1994-282270	A 19940729
			US 1995-483091	A 19950607
			US 1995-438091	A 19950607
			WO 1995-US8977	W 19950726

AB A method of preparing a stably **transformed**, transgenic cereal plant by microparticle bombardment of non-differentiated meristem cells from **immature embryos** at the early proembryo, mid proembryo, late proembryo, transitional or early coleoptilar stage is described. Transgenic sectors of the meristem tissue or cells fated to contribute to the meristem are then manipulated to enlarge transgenic sectors, either through selection and/or through effecting a proliferation from the tissue of shoots or multiple meristems per se. The shoot population is screened by a nonlethal enrichment assay, to identify either chimeric sectors that will contribute to germline transmission, or non-sectored, L2 periclinal chimeras that will by definition transmit to progeny. Increased time in culture, under selection, enhances the prospects for sectoral-to-periclinal conversions, and also selects for L1-to-L2 conversions which, through a shift in position, ultimately contribute to the germline. Transgenic sectors also are stabilized during the step of tillering. The use of a neomycin phosphotransferase gene in the selection of **transformed** tissue, and of a β -glucuronidase gene to identify transgenic tissue in plantlets is described. The formation of multiple meristems in tissue by mech. disruption of the apical dome increased the efficiency of **transformation**.

L3 ANSWER 47 OF 63 AGRICOLA Compiled and distributed by the National Agricultural Library of the Department of Agriculture of the United States of America. It contains copyrighted materials. All rights reserved.
(2005) on STN DUPLICATE 26

ACCESSION NUMBER: 97:4617 AGRICOLA
DOCUMENT NUMBER: IND20541778
TITLE: Production of transgenic **maize** plants and progeny by bombardment of Hi-II **immature embryos**.
AUTHOR(S): Songstad, D.D.; Armstrong, C.L.; Petersen, W.L.; Hairston, B.; Hinchee, M.A.W.
CORPORATE SOURCE: Monsanto, St. Louis, MO.
AVAILABILITY: DNAL (QK725.I43)
SOURCE: In vitro cellular & developmental biology. Plant : journal of the Tissue Culture Association, July/Sept 1996. Vol. 32, No. 3. p. 179-183
Publisher: Columbia, MD : Society for In Vitro Biology.
CODEN: IVCPEO; ISSN: 1054-5476
NOTE: Includes references
PUB. COUNTRY: Maryland; United States
DOCUMENT TYPE: Article
FILE SEGMENT: U.S. Imprints not USDA, Experiment or Extension
LANGUAGE: English

L3 ANSWER 49 OF 63 BIOSIS COPYRIGHT (c) 2005 The Thomson Corporation. on STN DUPLICATE 27

ACCESSION NUMBER: 1996:283604 BIOSIS
DOCUMENT NUMBER: PREV199699005960
TITLE: Analysis of the functional activity of the 1.4-kb 5'-region of the rice actin 1 gene in stable transgenic plants of **maize** (*Zea mays* L.).
AUTHOR(S): Zhong, Heng; Zhang, Shibo; Warkentin, Donal; Sun, Baolin; Wu, Tiyn; Wu, Ray; Sticklen, Mariam B. [Reprint author]
CORPORATE SOURCE: Dep. Crop Soil Sci., Dep. Entomol., 202 Pesticide Res. Cent., Michigan State Univ., East Lansing, MI 48824-1311, USA
SOURCE: Plant Science (Shannon), (1996) Vol. 116, No. 1, pp. 73-84.

CODEN: PLSCE4. ISSN: 0168-9452.

DOCUMENT TYPE: Article
LANGUAGE: English
ENTRY DATE: Entered STN: 25 Jun 1996
Last Updated on STN: 15 Aug 1996

AB The activity of the 5'-region of the rice actin 1 gene (Act1), covering a region 1.4 kb upstream of the Act1 translation initiation codon, was extensively analyzed in transgenic **maize** plants. The 5'-region of Act1 fused to the beta-glucuronidase (GUS) gene (gus) coding region was co-transformed to **maize** with the phosphinothricin acetyltransferase gene (bar) and the potato proteinase inhibitor II gene (pin2). One and 29 independent **transformation** events with expression of both bar and gus were recovered from bombardment of **immature embryo**-derived embryogenic callus of Hi-II derivative and bombardment of shoot tips of Honey N Pearl and Illinois Golden Extra Sweet, respectively. Expression of gus in tissues of transgenic plants was examined by histochemical assay, immunoblot analysis, and fluorometric GUS specific activity assay. A constitutive expression of the introduced gus was observed throughout the developmental stages of the vegetative and reproductive organs in transgenic **maize** plants. Quantitative analysis of GUS in transgenic plants showed that GUS, as percent of total soluble protein, was as much as 3.1% in leaves and 2.8% in roots. The functional activity of the 5'-region of Act1 was inherited to transgenic progeny. The results indicate that the 1.4-kb 5'-region of Act1 is an efficient and strong promoter for gene expression in stable transgenic **maize** plants.

L3 ANSWER 50 OF 63 CAPLUS COPYRIGHT 2005 ACS on STN

ACCESSION NUMBER: 1996:542941 CAPLUS
DOCUMENT NUMBER: 125:186964
TITLE: Three **maize** gene transfer techniques
AUTHOR(S): Wang, Guoying; Zhang, Hong; Ding, Qunxing; Dai, Jingrui; Xie, Youju
CORPORATE SOURCE: Coll. Biol., Beijing Agricultural Univ., Beijing, 100094, Peop. Rep. China
SOURCE: Shengwu Gongcheng Xuebao (1996), 12(1), 45-49
CODEN: SGXUED; ISSN: 1000-3061
PUBLISHER: Kexue
DOCUMENT TYPE: Journal
LANGUAGE: Chinese

AB The **maize transformation** was carried out through a bombarding particle gun, ultrasonication in a DNA buffer or ovary injecting by a microinjector. The plasmid pB480415, which carries a 3'-end truncated Bt-toxin protein gene and a hygromycin phosphotransferase (hpt) gene, was used in the **transformation**. Transgenic **maize** plants were obtained from **immature embryos** and embryogenic cells bombarded with the particle gun, embryogenic calli ultrasonicated under different conditions or ovaries injected after 10-20 h of pollination. All three techniques succeeded in gene transfer into **maize** cells.

L3 ANSWER 52 OF 63 BIOSIS COPYRIGHT (c) 2005 The Thomson Corporation. on STN DUPLICATE 28

ACCESSION NUMBER: 1995:355092 BIOSIS
DOCUMENT NUMBER: PREV199598369392
TITLE: Germline **transformation** of **maize** following manipulation of chimeric shoot meristems.
AUTHOR(S): Lowe, Keith [Reprint author]; Bowen, Ben; Hoerster, George; Ross, Margit; Bond, Diane; Pierce, Dottie; Gordon-Kamm, Bill
CORPORATE SOURCE: Pioneer Hi-Bred Internatl. Inc., 7300 NW 62nd Ave., PO Box 1004, Johnston, IA 50131, USA
SOURCE: Bio-Technology (New York), (1995) Vol. 13, No. 7, pp. 677-682.

CODEN: BTCHDA. ISSN: 0733-222X.

DOCUMENT TYPE: Article

LANGUAGE: English

ENTRY DATE: Entered STN: 30 Aug 1995

Last Updated on STN: 30 Aug 1995

AB Genetic **transformation** of **maize** has been limited to a small number of genotypes that form embryogenic tissue in culture. We have investigated whether cells in the developing shoot meristem of immature zygotic embryos might provide an alternative, more universal target for production of **transformed maize** plants. Following DNA delivery mediated by microprojectile bombardment, **immature embryos** developed into chimeric plants with transgenic sectors containing an antibiotic resistance marker and the beta-glucuronidase (GUS) gene at a high frequency. Because the majority of transgenic sectors were restricted in size, the probability of a **transformation** event contributing to the germline without further manipulation was low. To enlarge the transgenic sectors and increase the likelihood of germline transmission, the apical meristems of germinated plants were excised and cultured on cytokinin-containing medium with a selective agent. **Transformed** sectors were visualized by their non-bleached phenotype or by staining with a GUS histochemical stain. Hormonally-induced shoot multiplication produced plants with sectors that had a greater chance of contributing to the germline. Transmission to progeny was demonstrated both by transgene expression and by Southern analysis. This method has been used successfully with genotypes that include a sweet corn hybrid and an elite field corn inbred.

L3 ANSWER 53 OF 63 CAPLUS COPYRIGHT 2005 ACS on STN

ACCESSION NUMBER: 1996:699344 CAPLUS

DOCUMENT NUMBER: 125:319143

TITLE: Genetic modification of cereal crops by direct gene transfer

AUTHOR(S): Luetticke, S.; Becker, D.; Brettschneider, R.; Jaehne, A.; Loerz, H.

CORPORATE SOURCE: Angewandte Molekularbiologie der Pflanzen II, Universitat Hamburg, Hamburg, Germany

SOURCE: Induced Mutations and Molecular Techniques for Crop Improvement, Proceedings of an International Symposium on the Use of Induced Mutations and Molecular Techniques for Crop Improvement, Vienna, June 19-23, 1995 (1995), 389-397. International Atomic Energy Agency: Vienna, Austria.

CODEN: 63NLAP

DOCUMENT TYPE: Conference

LANGUAGE: English

AB On the basis of efficient in vitro culture and regeneration systems, reproducible **transformation** methods for different cereal crops were developed. Scutellar tissue of the **immature embryos** of hexaploid wheat and **maize** were used as targets for microprojectile mediated gene transfer. Bombardment of haploid microspores resulted in homozygous, transgenic and fertile barley plants. Each target was the subject of individual optimization processes of bombardment conditions by analyzing the transient β -glucuronidase activity. Furthermore, phosphinothricin resistance conferred by the bar gene turned out to be a suitable selectable marker for regenerating transgenic crop plants. Summarizing the results of independent **transformation** expts. for wheat and **maize** led to a **transformation** efficiency of one transgenic plant per 83 and 230 bombarded **immature embryos**, resp. For barley, the average of all the expts. was one transgenic plant per 2.8 ± 106 bombarded microspores. Primary **transformants** and progeny were analyzed for the enzyme activity of the two marker enzymes introduced and integration of the corresponding genes by Southern blot expts. Stable integration of the foreign DNA and its inheritance by progeny were

demonstrated. All the **transformed** plants showed normal morphol. and their development and flowering were comparable with those of seed derived plants.

L3 ANSWER 54 OF 63 BIOSIS COPYRIGHT (c) 2005 The Thomson Corporation. on
STN DUPLICATE 29

ACCESSION NUMBER: 1996:15234 BIOSIS
DOCUMENT NUMBER: PREV199698587369
TITLE: Biolistic introduction of a synthetic Bt gene into elite
maize.
AUTHOR(S): Hill, M.; Launis, K.; Bowman, C.; McPherson, K.; Dawson,
J.; Watkins, J.; Koziel, M.; Wright, M. S.
CORPORATE SOURCE: Ciba Biotechnol., P.O. Box 12257, Research Triangle Park,
NC 27709-2257, USA
SOURCE: Euphytica, (1995) Vol. 85, No. 1-3, pp. 119-123.
CODEN: EUPHAA. ISSN: 0014-2336.
DOCUMENT TYPE: Article
LANGUAGE: English
ENTRY DATE: Entered STN: 12 Jan 1996
Last Updated on STN: 12 Jan 1996

AB A synthetic Bt gene encoding a truncated version of the CryIA(b) protein derived from *Bacillus thuringiensis* was successfully introduced into elite **maize** using microprojectile bombardment of **immature embryos**. The method used to initiate and identify **transformation** events is described. We describe the detailed parameters used for the Biolistics device as well as the plasmids used for the **transformations**. The plasmids contained the synthetic Bt gene driven by either the 35S CaMV promoter or a combination of two tissue-specific promoters, leaf and pollen, derived from **maize**. Specific conditions for the culture of Type I callus from **immature embryos**, the phosphinothricin (PPT) selection protocol, and the regeneration of plants are discussed. T0 and T1 plants were initially identified using the pH-dependent chlorophenol red test and/or the histochemical beta-glucuronidase (GUS) assay. PCR and Southern data confirm the presence of the 35S CaMV promoter and the synthetic Bt gene.

L3 ANSWER 58 OF 63 AGRICOLA Compiled and distributed by the National Agricultural Library of the Department of Agriculture of the United States of America. It contains copyrighted materials. All rights reserved.
(2005) on STN DUPLICATE 33

ACCESSION NUMBER: 94:4165 AGRICOLA
DOCUMENT NUMBER: IND20363068
TITLE: Enhanced GUS gene expression in cereal/grass cell suspensions and **immature embryos** using the **maize** ubiquitin-based plasmid pAHC25.
AUTHOR(S): Taylor, M.G.; Vasil, V.; Vasil, I.K.
AVAILABILITY: DNAL (QK725.P54)
SOURCE: Plant cell reports, 1993. Vol. 12, No. 9. p. 491-495
Publisher: Berlin, W. Ger. : Springer International.
CODEN: PCRPD8; ISSN: 0721-7714
NOTE: Includes references
PUB. COUNTRY: Germany
DOCUMENT TYPE: Article
FILE SEGMENT: Non-U.S. Imprint other than FAO
LANGUAGE: English

AB Transient GUS (beta-glucuronidase) expression was visualized in cell suspensions of *Triticum aestivum*, *Zea mays*, *Pennisetum glaucum*, *Saccharum officinarum*, *Pennisetum purpureum* and *Panicum maximum* after microprojectile bombardment with pBARGUS and pAHC25 plasmid DNAs. pBARGUS contains the GUS (Uida) gene coding region driven by the Adh1 promoter and the Adh1 intron 1, as well as the BAR gene coding region driven by the CaMV 35S promoter and the Adh1 intron 1. pAHC25 contains the GUS and BAR gene coding regions driven by the **maize** ubiquitin promoter,

first exon and first intron (Ubil). The effectiveness of the constructs was first compared in cell suspension cultures by counting blue expression units (b.e.u.). The expression of construct pAHC25 ranged from 3 to 50 fold greater than pBARGUS in different species. In addition, the two plasmids were quantitatively compared in *Triticum aestivum* and *Zea mays* by using the more sensitive GUS fluorometric assay to determine the amount of methylumbelliferylferide (MU) produced. There was more than a 30 fold increase in MU production with pAHC25 than with pBARGUS in the wheat suspension, while the **maize** suspension showed only a 2.5 fold increase with the pAHC25 construct. Transient GUS expression was also visualized in **immature embryos** of *Pennisetum glaucum* following bombardment with pBARGUS and pAHC25 DNA. Expression of plasmid pAHC25 was twice as high as pBARGUS. A comparison of two DNA/gold preparation methods, as well as repeated sonications of the DNA/gold mixture, had no effect on the number of b.e.u.

L3 ANSWER 61 OF 63 BIOSIS COPYRIGHT (c) 2005 The Thomson Corporation. on STN DUPLICATE 36

ACCESSION NUMBER: 1991:364488 BIOSIS
DOCUMENT NUMBER: PREV199192052713; BA92:52713
TITLE: TRANSIENT TRANSFORMATION OF MAIZE
TISSUES BY MICROPARTICLE BOMBARDMENT.
AUTHOR(S): REGGIARDO M I [Reprint author]; LUIS ARANA J; ORSARIA L M;
PERMINGEAT H R; SPITTELER M A; VALLEJOS R H
CORPORATE SOURCE: CEFOTBI, SUIPACHA 531, 2000 ROSARIO, ARGENTINA
SOURCE: Plant Science (Shannon), (1991) Vol. 75, No. 2, pp.
237-244.
CODEN: PLSCE4. ISSN: 0168-9452.
DOCUMENT TYPE: Article
FILE SEGMENT: BA
LANGUAGE: ENGLISH
ENTRY DATE: Entered STN: 13 Aug 1991
Last Updated on STN: 13 Aug 1991

AB Transient **transformation** of **maize** coleoptiles with the β -glucuronidase gene was achieved with a particle gun built by us essentially according to the concept of Klein et al. (Nature, 327 (1987) 70-73). Either tungsten or gold particles were used to **transform** coleoptiles. **Transformed** blue cells were found in different cell layers and occasionally in as deep as the 8th layer. A **transformation** efficiency of up to 2% was obtained when the gene was in plasmid pAI1GUS driven by the alcohol dehydrogenase promoter plus the intron 1. The 35S promoter of cauliflower mosaic virus (plasmid pBI221) was ten times less efficient. Other **maize** tissues like **immature embryos**, leaf basal segments and cell suspensions were also **transformed** but at lower rates. The time course of the histochemical reaction for the β -glucuronidase activity showed that the number of **transformed** blue cells detected increased up to 72 h of starting the reaction. It is concluded that **transformation** of **maize** coleoptiles with the particle gun is a good method for testing gene expression in transient systems.

L3 ANSWER 62 OF 63 CAPLUS COPYRIGHT 2005 ACS on STN

ACCESSION NUMBER: 1993:618565 CAPLUS
DOCUMENT NUMBER: 119:218565
TITLE: Study of the transient **transformation** of **maize** tissue by bombardment with microparticles with high density
AUTHOR(S): Arana, Jose Luis; Reggiardo, Martin I.; Permingeat, Hugo R.; Spitteler, Marcelo A.; Lopez, Monica E.; Orsaria, Lelia M.; Vallejos, Ruben H.
CORPORATE SOURCE: Cent. Estud. Fotosint. Bioquim., UNR, Rosario, 2000, Argent.
SOURCE: Anales de la Academia Nacional de Ciencias Exactas, Fisicas y Naturales (Buenos Aires) (1990), 42, 243-50

DOCUMENT TYPE: Journal

LANGUAGE: Spanish

AB A system for direct gene transfer into **maize** tissues by means of microprojectile acceleration is described. The authors report the transient **transformation** of coleoptiles, **immature embryos**, base leaf segments and cell suspension cultures. Two different gene constructions were used: plasmids pAI1GUS and pBI221. A high **transformation** efficiency (0.9-2%) was achieved in coleoptile tissues when bombardment conditions were optimized. Coleoptile **transformation** seems to be a good model system to study gene expression in **maize**. The **transformation** of embryogenic cell suspension cultures could lead to transgenic **maize** plants.

=>

Analysis of the functional activity of the 1.4-kb 5'-region of the rice actin 1 gene in stable transgenic plants of maize (*Zea mays* L.)

Heng Zhong^a, Shibo Zhang^a, Donal Warkentin^a, Baolin Sun^a, Tiyun Wu^b, Ray Wu^b,
Mariam B. Sticklen^{*a}

^aDepartment of Crop and Soil Sciences, Department of Entomology, 202 Pesticide Research Center, Michigan State University,
East Lansing, MI 48824-1311, USA

^bSection of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853, USA

Received 24 May 1995; revision received 24 January 1996; accepted 25 January 1996

Abstract

The activity of the 5'-region of the rice actin 1 gene (*Act1*), covering a region 1.4 kb upstream of the *Act1* translation initiation codon, was extensively analyzed in transgenic maize plants. The 5'-region of *Act1* fused to the β -glucuronidase (GUS) gene (*gus*) coding region was co-transformed to maize with the phosphinothricin acetyltransferase gene (*bar*) and the potato proteinase inhibitor II gene (*pin2*). One and 29 independent transformation events with expression of both *bar* and *gus* were recovered from bombardment of immature embryo-derived embryogenic callus of Hi-II derivative and bombardment of shoot tips of Honey N Pearl and Illinois Golden Extra Sweet, respectively. Expression of *gus* in tissues of transgenic plants was examined by histochemical assay, immunoblot analysis, and fluorometric GUS specific activity assay. A constitutive expression of the introduced *gus* was observed throughout the developmental stages of the vegetative and reproductive organs in transgenic maize plants. Quantitative analysis of GUS in transgenic plants showed that GUS, as percent of total soluble protein, was as much as 3.1% in leaves and 2.8% in roots. The functional activity of the 5'-region of *Act1* was inherited to transgenic progeny. The results indicate that the 1.4-kb 5'-region of *Act1* is an efficient and strong promoter for gene expression in stable transgenic maize plants.

Keywords: Gene expression; β -Glucuronidase (GUS); Rice actin 1 gene (*Act1*); Transgenic maize; Shoot meristem

1. Introduction

The regulatory activity of the 5' region of the rice actin 1 gene (*Act1*), when fused to the bacterial

β -glucuronidase (GUS) gene (*gus*), has been extensively analyzed in transgenic rice plants [1]. This *Act1* 5' region covers a region 2.1 kb upstream of the translation initiation codon of the *Act1* gene, including a 1.64 kb nontranscribed 5' flanking sequence and several *Act1* transcribed sequences. The *Act1*-*Gus* gene fusion was constitutively expressed throughout the sporophytic and gametophytic tissues in transgenic rice. The tissue of transgenic rice plants had as much as 3% of total soluble protein as β -glucuronidase. Although this fusion construct has also been introduced into

Abbreviations: BA, N⁶-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; G, glufosinate ammonium; GUS, β -glucuronidase; Hi-II, type II embryogenic callus line of A188 \times B73; HNP, Honey N Pearl; IBA, indole-3-butyric acid; IGES, Illinois Golden Extra Sweet; MS, Murashige and Skoog (1962) basal medium; N6, Chu et al. (1975) rice anther culture medium; SDS, sodium dodecyl sulfate.

* Corresponding author.

barley [2], wheat [3,4], and tritordeum [5], the expression of the GUS gene under the *Act1* 5'-region in these transgenic plants was not analyzed in as much detail as in transgenic rice [1].

The characterization of the rice *Act1* 5' region revealed that the minimum amount of *Act1* sequence required for maximal GUS expression is a 0.83 kb nontranscribed 5' flanking sequence and the several *Act1* transcribed sequences, covering a region 1.4 kb upstream of the *Act1* translation initiation codon, in transient assays of transformed rice protoplast [6]. Similar analysis of the activity of this *Act1-Gus* gene fusion has been reported in rice, barley and maize cells at the transient gene expression level, and it was shown to be much higher than that of the CaMV 35S promoter [7,8]. Previously, we have transformed turfgrass with this 1.4-kb 5' region of rice *Act1* and demonstrated the high level of constitutive expression of *Act1-Gus* gene fusion in the vegetative parts of transgenic turfgrass plants [9].

Here we report the extensive analysis of the functional activity of the 1.4-kb 5'-region of the rice *Act1* in stable transgenic plants of maize. In our work, the level of expression of the introduced *gus* driven by the 1.4-kb rice *Act1* 5'-region was analyzed in shoot and floral meristems, young leaves and leaf bases of mature leaves, roots, pollen grains, and zygotic embryos. Our study indicated that the region 1.4 kb upstream of the *Act1* translation initiation codon is a strong regulator in stable transgenic maize plants.

2. Materials and methods

2.1. Production of transgenic plants with *Act1-Gus* fusion

Two transformation systems including the bombardment of shoot tips and the bombardment of immature embryo-derived callus were performed.

We have reported the development of a system for transformation of maize via bombardment of shoot tips [10]. In summary, multiple shoot-tip cultures of two sweet corn genotypes, Honey N Pearl (HNP) and Illinois Golden Extra Sweet (IGES), were initiated on MS basal medium [11] containing 2 mg/l N_6 -benzyladenine (BA) and 0.5

mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) from the mature kernels as described previously [12]. Shoot tips or shoot-tip clumps were positioned in an area of about 1.5 cm at the center of a Petri dish containing phytagel-solidified culture medium and bombarded 4 times with 1.0 μ m tungsten particles at a density of 75 μ g/shot and an acceleration pressure of 1550 psi.

For transformation via bombardment of embryogenic callus, the type II embryogenic callus was initiated from immature embryos of field plants germinated from seeds. The seeds were from self-pollinated greenhouse plants that were regenerated from embryogenic callus line Hi-II [13]. Samples of fresh embryogenic callus, a thin layer ~3 cm in diameter, were arranged at the center of Petri dishes and were bombarded as previously reported [14,15].

The plasmid p*Act1*-F (Fig. 1a) [6] was delivered into cells of embryogenic callus and shoot tips in co-transformation with plasmid pTW-a (Fig. 1b) in a 1:1 ratio. The p*Act1*-F contains *Act1-Gus* gene fusion. The pTW-a contains two linked genes, the potato proteinase inhibitor II gene (*pin2*) [16] driven by *pin2* promoter with *Act1* 5' intron [6] and the *Streptomyces hygroscopicus bar* gene [17] driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter.

The bombarded shoot-tips were multiplied on MS basal medium plus 2 mg/l BA and 0.5 mg/l 2,4-D for 4 weeks, and then were selected on the same medium with 3 mg/l glufosinate ammonium (G) for 4 weeks and with 5 mg/l G at 4-week intervals for another 4 months. Plantlets were subsequently regenerated from G-resistant shoot-tip clumps on MS medium containing 0.5 mg/l BA, 0.5 mg/l indole-3-butyric acid (IBA) and 5–10 mg/l G and MS medium containing 1 mg/l IBA and 10 mg/l G.

The bombarded callus samples were grown for 1 week on N6 medium [18] supplemented with 3.0 mg/l L-proline and 2.0 mg/l 2,4-D (Sigma). They were then selected on the same medium supplemented with 3 mg/l G for ~2 weeks, followed by another 4-week selection on the medium containing 5 mg/l G. The selected G-resistant colony was transferred to embryogenesis medium [19] for 2 weeks. The somatic embryos were germinated on MS basal medium plus 2% sucrose. The regener-

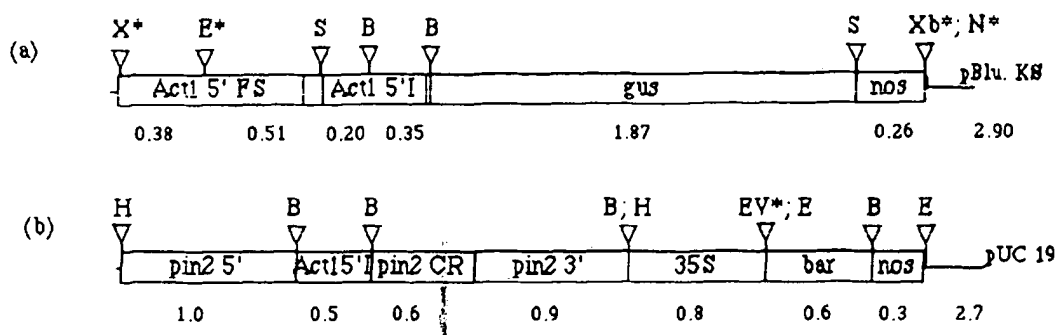


Fig. 1. Restriction maps of constructs pAct1-F and pTW-a. (a) pTW-a contains two linked genes: (1) the *pin2* coding sequence (*pin2*) under the *pin2* promoter (*pin2* 5') with *Act1* intron (*Act1* I), plus *pin2* terminator (*pin2* 3'); (2) *bar* is *bar* coding sequence (*bar*) under CaMV 35S promoter (35 S), plus the *nos* terminator (Nos). (b) pAct1-F contains the *Act1* 5'-flanking sequence (*Act1* FS), 5'-noncoding exons of *Act1* (open boxes), *Act1* 5'-intron (*Act1* I), the *gus* coding sequence (*gus*), and the *nos* terminator (*nos*). Abbreviations: B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; N, *Not*I; S, *Sac*I; X, *Xho*I; and Xb, *Xba*I. *Unique site.

ated plantlets were selected again in Magenta boxes containing the above regeneration medium with 5 mg/l of G.

All of G-resistant plantlets with 2–3 leaves were transferred to pots containing soil in the greenhouse.

A 1% solution of Ignite® non-selective herbicide (AKA BASTA, HOE-39866, Hoecht-Roussel Agri-Vet Company, Somerville, NJ) containing 2 g/l of G, the active ingredient, was sprayed on transgenic plants following an earlier report [14].

Ten µg of genomic DNA isolated from leaves of greenhouse-grown plants were digested overnight, electrophoresed on 0.8% agarose gels, and transferred to nylon membranes [20]. The blots were hybridized with probes of *bar* (0.6 kb *bar* coding sequence), *pin2* (1.5 kb containing *pin2* coding region and *pin2* terminator), and *gus* (1.87 kb *gus* coding sequence) (Fig. 1a and b) for analysis of the presence of the *bar*, *pin2*, and *gus* in transgenic plants.

2.2. Histochemical GUS staining

Roots, shoot tips and leaves of in vitro-grown transgenic plants at the 4–6 leaf stage were collected and stained in GUS staining buffer with X-glucuronide (X-gluc) at 37°C for the histochemical assay [21]. Male and female floral meristems were isolated from greenhouse-grown transgenic plants.

Pollen grains were isolated from mature and immature anthers of greenhouse-grown transgenic plants. All of these tissues and organs were stained with X-gluc. Thin cross-sections of roots, leaves, and male and female flowers were made by hand. The immature embryos were isolated ~15–20 days after pollination and stained with X-gluc. The immature embryos were also germinated in vitro to produce T1 plants for histochemical GUS staining.

2.3. Immunoblot analysis

Transgenic plants were assayed for gene expression by immunoblot analysis [22]. Approximately 20 mg fresh weight of each tissue of a transgenic plant was ground in 200 µl 2 × SDS gel-loading buffer (100 mM Tris-HCl, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol, pH 6.8) containing 14 mM β-mercaptoethanol and 100 mM phenylmethylsulfonyl fluoride, then centrifuged at 14 000 rev./min for 10 min at 4°C. An extract from an untransformed plant was used as the negative control. Purified β-glucuronidase (1.0 µg and 0.1 µg) was used as the positive control. Rainbow pre-stained protein molecular weight markers (Amersham International Inc.) were used. A 20 µl aliquot of each sample extract was boiled at 100°C for 5 min, loaded onto a 10% running, 6% stacking SDS-polyacrylamide gel [23], electro-

phoresed at 30 mA for ~1 h, and electroblotted overnight onto a 0.45 μ m nitrocellulose membrane [24]. The membrane was incubated in rabbit anti- β -glucuronidase antiserum (Clontech) diluted 1:800, followed by alkaline phosphatase conjugated goat anti-rabbit IgG antiserum at 1:7500 dilution. The membrane was incubated with the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate [9].

2.4. Densitometer scanning of Coomassie stained gels

Coomassie-stained SDS gels were used to quantify the β -glucuronidase as percent of total soluble protein extracted from each tissue. The SDS gels were loaded with samples, electrophoresed, and stained in Coomassie brilliant blue staining solution for 4 h. The gels were destained for ~24 h. The 68 kD β -glucuronidase band present in the putative transgenic extracts had similar mobility to the purified β -glucuronidase positive control. The same size band was not visible in the untransformed plant sample. Quantification of the β -glucuronidase as percent of total soluble protein was made by using the AMBIS densitometer computer program.

2.5. Fluorometric assay

GUS specific activity was measured by fluorometric assay [21]. Total soluble protein from each tissue was extracted in 200 μ l GUS extraction buffer. Protein concentration of the samples was determined by the dye-binding method of Bradford [25] with a Bio-Rad kit. All of the samples were adjusted to the same concentration of total soluble protein before assay. A 50 μ l aliquot of each sample extract was added to 500 μ l of methylumbelliferyl- β -D-glucuronide (MUG) buffer (1 mM MUG in GUS extraction buffer), and then incubated at 37°C for 0, 1, 2, 4 and 17 h. A standard curve was established with methylumbelliferone (MU). Fluorescence was measured and analyzed with the AMBIS densitometer computer program, and the concentrations of methylumbelliferone were determined by regression analysis versus the standard curve.

3. Results

3.1. Production of transgenic plants with the *Act1-Gus* gene fusion

The *Act1-Gus* gene fusion was co-transformed with *bar* and *pin2* into maize plants by bombardment of shoot tips and embryogenic callus. We have reported 29 independent transformed shoot-tip clumps of both HNP and IGES with intensive GUS-staining which were isolated on the selection of 3–5 mg/l G 3 months after bombardment [10]. Southern blot analysis of uncut genomic DNA from each of these independent transformation events showed the presence of all transgenes, *bar*, *pinII* and *gus* [10]. An intensely GUS-stained callus colony was also recovered 3 months after the bombardment of embryogenic callus line Hi-II under the selection of 3–5 mg/l G. All of regenerated plantlets were resistant to 5 mg/l G in vitro, a level which killed 100% of untransformed plantlets. More than 1500 plantlets regenerated from 10 independent transgenic shoot-tip clumps of both HNP and IGES were transferred to the greenhouse. A total of 197 putative transgenic plantlets regenerated from the callus colony of Hi-II (MT1-MT197) were also transferred to the greenhouse. Untransformed plants and the putative transgenic plants were sprayed with 1% herbicide Ignite® solution. All of the sprayed putative transgenic plants survived and displayed no necrotic symptoms, while all untransformed plants were killed within 7 days.

Preliminary southern blot analysis of genomic DNA of five plants from each of the independent transformed events revealed that the five plants from the same event were regenerated from the same transformed cell (data not shown). The insertion site of the transgenes, *bar* and *gus*, in the leaf genomic DNA of greenhouse-grown transgenic plants from five independent transformation events of all three genotypes was compared (e.g. see Fig. 2). The results showed that the transgenes were integrated into the genomes of the transgenic plants. A 3.3 kb *gus*-hybridized band, containing a portion of *Act1* promoter, the *gus* and *nos* 3' terminator in pAct1-F, and a 0.9 kb *bar*-hybridized band, containing the *bar* and *nos* 3' terminator in

pTW-a, were observed in all transgenic plants after digestion with *EcoRI/NotI* (Fig. 2A) or *EcoRI* (Fig. 2B), respectively. The band pattern of genomic DNA digested with *EcoRI* (Fig. 2A, a unique site in pAct1-F) or *EcoRV* (Fig. 2B, a unique site in pTW-a) indicated that both *gus* and *bar* were integrated into different sites of genomic DNA with various copy numbers in different transformation events.

3.2. Histochemical GUS staining of tissues and organs of transgenic T0 and T1 plants

The roots of more than 200 in vitro-grown control and independently transformed plants from all three genotypes were assayed by incubation in X-

gluc GUS staining solution for 30 min to 4 h at 37°C. Intense GUS staining was observed in the root tip and elongation zone of all of the primary roots and adventitious roots in more than 93% of transgenic plants examined but not in control plants (Fig. 3a and b). Root hairs, which were the tissue that responded to GUS staining most rapidly, turned blue 10–30 min after staining. Less than 6% of transgenic plants showed higher GUS activity only in the vascular tissues at the elongation zones (Fig. 3a and c). It was apparent that the GUS staining was much more intense in the cells of root hairs, epidermal layers, endodermis, and vascular areas, but was much lower in cortex cells (Fig. 3d).

When leaves and shoot tips of in vitro-grown

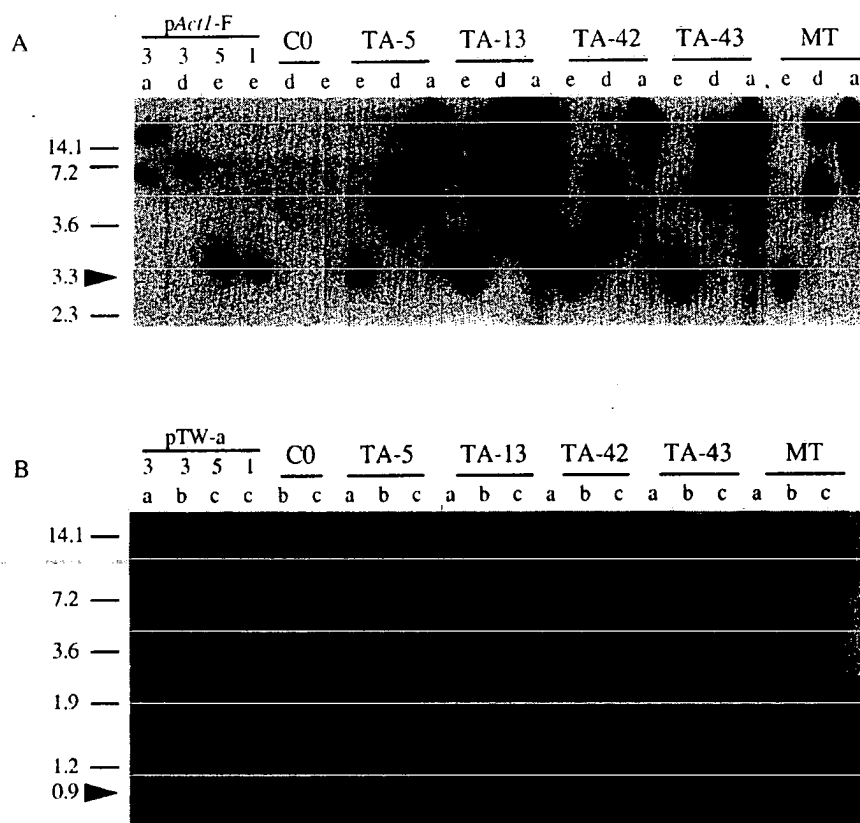


Fig. 2. Southern blot analysis of transgenic T0 plants harboring the transgenes *gus* (A) and *bar* (B). The amount of plasmid DNA represented 1, 3 and 5 copies in plant genomic DNA. Each lane contains 10 µg of genomic DNA from control plant (CO, HNP) or transgenic maize plants of maize (T0: TA-5 and TA-13 of HNP, TA-42 and TA-43 of IGES, and MT of Hi-II). Lane a, undigested DNA; lane b, *EcoRV*-digested DNA; lane c, *EcoRI*-digested DNA; lane d, *HindIII*-digested DNA, and lane e, *EcoRI/NotI*-digested DNA. Filters were hybridized with α -³²P-labeled *gus* or *bar* coding region probe, respectively.

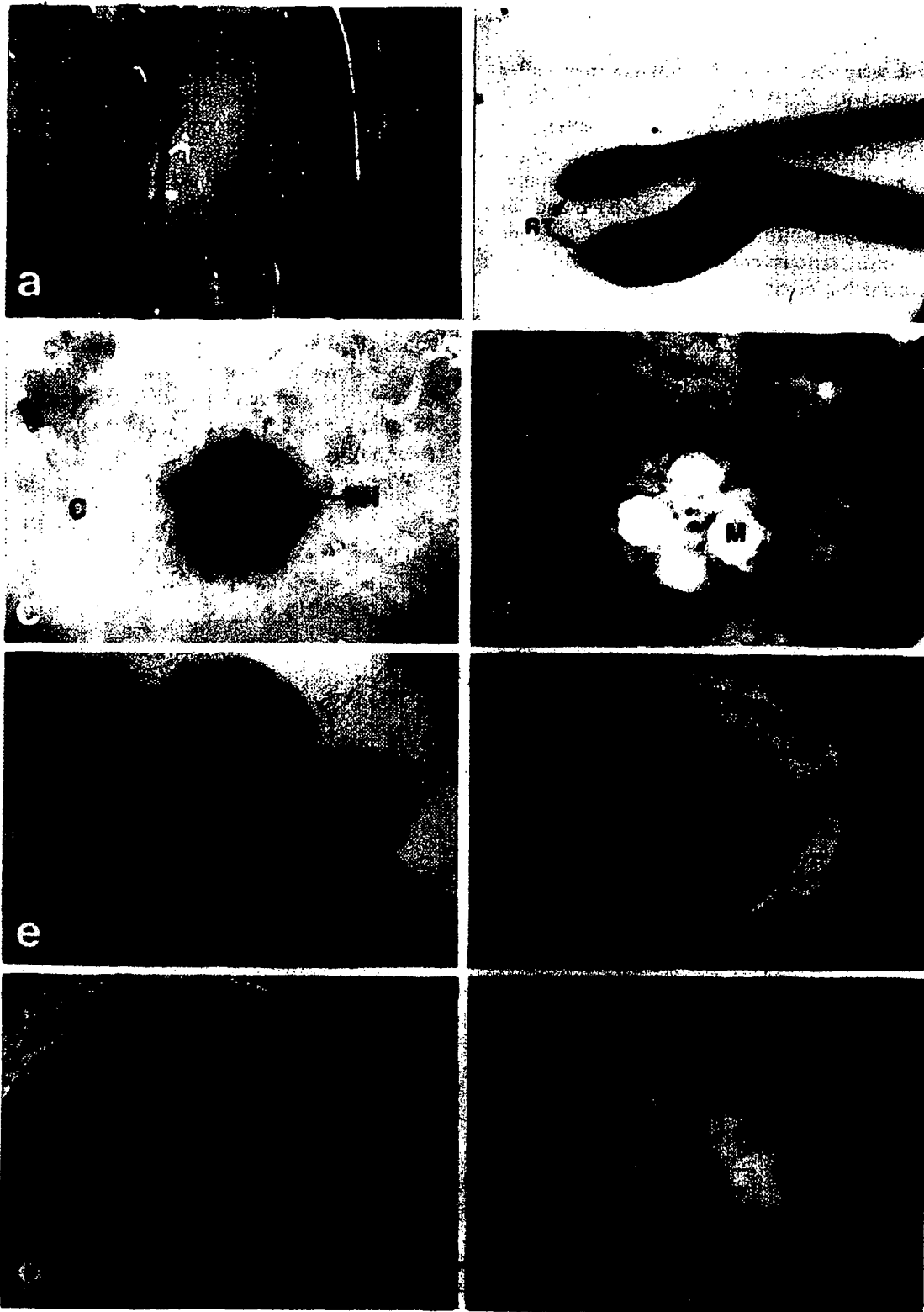


Fig. 3. Histochemical assay of *gus* expression in tissues of transgenic maize with *pAct1-F*. (a) Roots from control (CR) and transgenic plants; PR, primary roots; AR, adventitious roots; SR, secondary roots; V, expression only in the vascular tissue. (b) *gus* expression in the root tips (RT). (c) Cross-section of a root with the expression only in the vascular tissues but not in cortex cells; M, metaxylem; PH, phloem. (d) Cross-section of a primary root; C, cortex cells; E, epidermis cells; EN, endodermis cells; M, metaxylem; PH, phloem; PX, protoxylem; RH, root hairs. (e) A shoot tip. (f) Cross-section of leaf sheathes; LV, lateral vein; IV, intermediate vein. (g) An enlarged cross-section of a leaf blade. (h) Cross-section of a stem proximal to the leaf base in the node; AX, axillary bud; LS, leaf sheath; VB, vascular bundle. (i) Cross-section of a stem-axillary bud junction. (j) A tassel primordium. (k) Cross-section of an immature tassel; SP, spikelet primordium. (l) A developing tassel; GO, out glume. (m) Different development stages of ear; SP, spikelet primordium; SL, silk. (n) Cross-section of a developing ear; SL, silk. (o) Immature pollen grains. (p) Immature embryos.

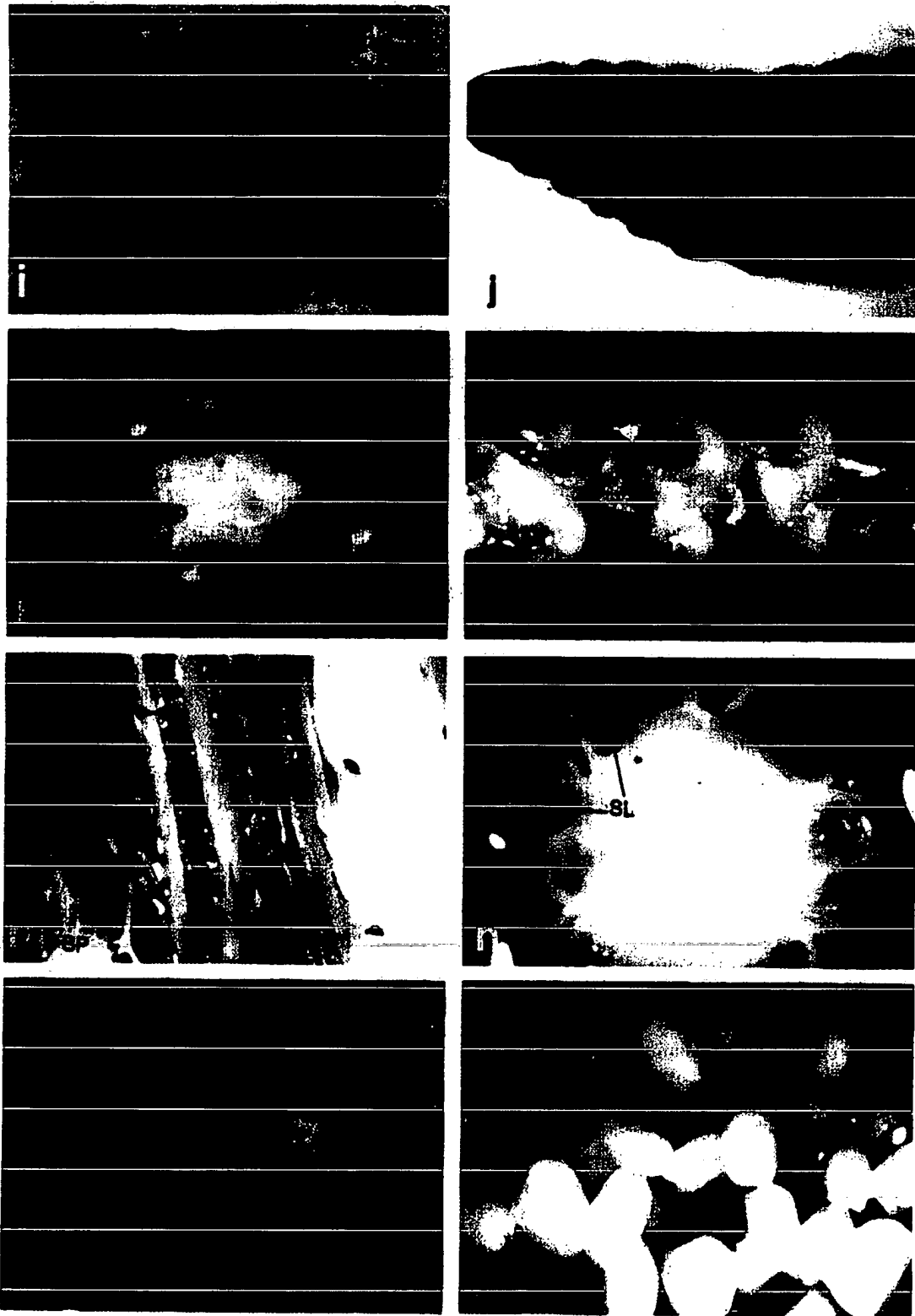


Fig. 3. (continued)

and greenhouse-grown transgenic and control plants were assayed with X-gluc, the entire shoot tips (Fig. 3e) and the youngest leaves (Fig. 3f) showed most intense GUS staining in all plants tested. The older leaves of the most in vitro- and greenhouse-grown plants had high GUS activity at the leaf bases and several expressing sectors within the center of leaves or at the leaf tips. Thin cross-sections of GUS-stained leaf bases showed that high-level GUS expression was located in epidermal cells and vascular tissue, while the expression was lower in mesophyll cells (Fig. 3g). The axillary buds and the vascular tissues in the internodes presented a high intensity of GUS staining (Fig. 3h and i). The vascular tissues proximal to leaf base in the node showed the intense blue color.

Young male and female florescences of transgenic plants stained with X-gluc showed high GUS activity in spikelet primordia (Fig. 3j, k and l). Most of the more-developed florets showed a higher level of GUS activity in inner and outer glumes and anthers. In an exception observed, the high level of GUS activity displayed only in the developing anther primordia but not in the inner and outer glumes (Fig. 3m). The developing silks of transgenic plants showed high GUS activity (Fig. 3n and o), while the activity was very low or not visible in the highly developed silks and floral tissues (Fig. 3l). Pollen grains collected from most transgenic plants of all three genotypes showed an approximate 1:1 positive:negative GUS staining ratio with a variable GUS activity (Fig. 3o).

The intensity of GUS staining in the immature embryos of transformants depended on their developmental stage (Fig. 3p). The younger the immature embryos, the more intense the blue. The statistic analysis of results from GUS assay of immature embryo, young leaf section, root and pollen grain of T1 and T2 plants revealed that the segregation of GUS expression followed Mendelian inheritance in the T2 progeny tested but not in all of the T1 progeny tested (3:1 for self-pollination and 1:1 for cross-pollination with a single gene in a single locus) (data not shown). Southern blot analysis of the leaf genomic DNA of selected GUS-positive T1 and T2 plants confirmed the presence of *gus* in the T1 and T2 progeny (data not shown).

3.3. Immunoblot analysis of transgenic plant tissues

Protein samples extracted from leaves (first, fourth, sixth) and three individual roots of a single transgenic plant of independent transformation events derived from HNP, IGES and callus line Hi-II were analyzed by immunoblot. The 68 kD band of β -glucuronidase was present in samples from transgenic leaves and roots (e.g. see Fig. 4, lanes 1–6), but not in the sample from an untransformed plant (Fig. 4, lane 7). In another experiment, equal amounts of three root segments that showed three levels of GUS staining in a single stained root (dark blue, light blue and no visible blue) were tested by immunoblot. The result showed that the lane loaded with a sample from a dark blue segment had a more intense β -glucuronidase band than that from a light blue segment, and there was no visible band from a segment with no visible blue color (data not shown). This indicated that histochemical GUS staining was consistent with immunoblot for analysis of β -glucuronidase.

3.4. Quantitative analysis of GUS protein by densitometer scanning of Coomassie-stained SDS gels

Densitometer scanning of Coomassie-stained SDS gels containing the same protein samples extracted for immunoblot analysis as above was done to quantify β -glucuronidase as percent of total soluble protein. The level of β -glucuronidase in total soluble protein of the transgenic leaves and roots can be as much as 2.2% and 2.6% for Hi-II, 3.1% and 2.8% for HNP, or 2.9% and 2.5% for IGES, respectively (Fig. 5). The *Act1-Gus* expression level in roots was more consistent than that in leaves among different genotypes. The younger the leaf, the higher the level of GUS.

3.5. Fluorometric assay

As compared to GUS specific activity measured by fluorometric assay in control plants of comparable age, the activity in the leaves and roots of the transgenic plants derived from HNP, IGES and Hi-II was more than 500-fold higher (Table 1). Transgenic roots had relatively higher specific

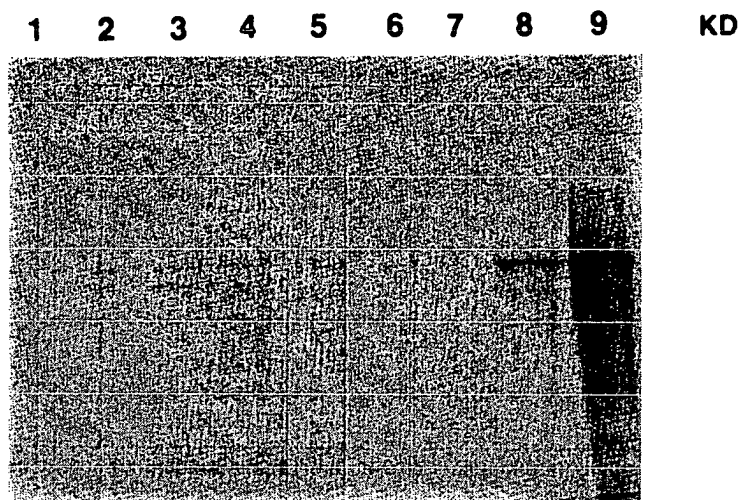


Fig. 4. Immunoblot analysis of roots and leaves of maize transgenic T0 plants with *pAct1-F*. Lanes 1–3, first, second and third root samples from a transgenic plant MT 228; lanes 4–6, sixth, fourth and first leaf samples of the transgenic plant MT 228; lane 7, leaf sample of an untransformed plant; lanes 8–9, 0.1 and 1.0 μ g of purified β -glucuronidase. The arrow indicates the size of 68 kD β -glucuronidase.

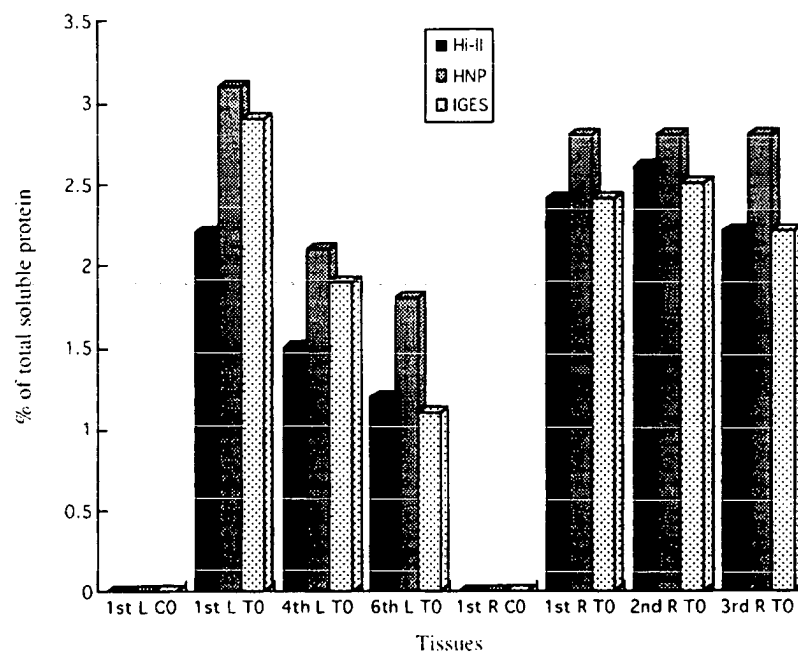


Fig. 5. Quantification of GUS in the tissues of transgenic maize plants contained *pAct1-F*; C0, control plants; T0, primary transformants; 1st L, the youngest, fully expanded leaf; 1st R, primary root.

Table 1

Fluorometric assay of β -glucuronidase specific activity in the primary transgenic maize plants (T0) contained pAct1-F^a

Tissues	Hi-II	HNP	IGXS
Control 1st leaf	0 \pm 14	0 \pm 9	0 \pm 27
T0 1st leaf	545 \pm 141	630 \pm 97	601 \pm 144
T0 2nd leaf	418 \pm 211	585 \pm 184	548 \pm 48
T0 3rd leaf	126 \pm 60	503 \pm 53	477 \pm 92
T0 4th leaf	459 \pm 153	431 \pm 184	501 \pm 139
T0 5th leaf	92 \pm 12	407 \pm 49	322 \pm 56
T0 6th leaf	315 \pm 92	373 \pm 126	265 \pm 115
Control 1st root	0 \pm 21	0 \pm 15	0 \pm 8
T0 1st root	522 \pm 188	555 \pm 47	586 \pm 118
T0 2nd root	539 \pm 181	593 \pm 42	545 \pm 60
T0 3rd root	516 \pm 121	501 \pm 93	577 \pm 157

^aThe values are the mean-specific activity (nmol/mg/min) of β -glucuronidase \pm standard error; first leaf is the youngest, fully expanded leaf; and first root is the primary root.

GUS activity and less variability than transgenic leaves. Among transgenic leaves, the youngest, fully expanded leaf (1st leaf) showed higher specific activity than older leaves. These results were consistent with the histochemical GUS staining assay and quantification analysis of β -glucuronidase described above.

4. Discussion

Here we report in detail that the 1.4-kb 5'-region of rice *Act1* promoted a high-level, constitutive expression of the introduced β -glucuronidase gene throughout the development of stable transgenic maize plants of three genotypes. The functional activity of the *Act1-GUS* gene fusion was similar in the stable transgenic maize plants of three genotypes derived from the bombardment of shoot tips and embryogenic callus. Based upon our result, and in combination with the earlier transient gene expression studies of *Act1*-based vectors in maize [7,8], it appears that the 5'-region of *Act1* is an efficient and strong promoter for gene expression in stable transgenic maize plants. Transgenic maize plants have been reported to express GUS activity under the CaMV 35S promoter together with the maize *adh1* intron in herbicide-resistant callus lines and in leaf tissues of transgenic plants [14]. However, it was not pos-

sible to make a direct comparison between the CaMV 35S and *Act1* promoters, because the two constructs had different introns. It was shown that the 5'-region of rice *Act1* was active in all cell types of transgenic rice plants [1], but the CaMV 35S promoter was not [26,27].

The promoter activity of the rice 1.4-kb *Act1* 5' region in our transgenic maize plants was comparable to its activity in transgenic turfgrass [9] and the activity of the rice 2.1-kb *Act1* 5'-region in other transgenic crops [1,2,4,5]. High activity of the rice 1.4-kb *Act1* 5' region was in all actively growing tissues, including shoot and floral meristems, young leaves, bases of mature leaves, root tips, pollen grains and immature embryos, of transgenic maize plants of all three genotypes tested. In transgenic rice, high GUS activity was observed in leaf intercalary meristems, axillary buds, and trichomes of leaves [1]. Pollen grains and zygotic embryos in our transgenic maize had intense GUS staining, as was observed in other transgenic crops [1,2,4,5].

Up to 3.1% and 2.8% of the total soluble protein in leaf and in root, respectively, of transgenic maize plants was β -glucuronidase, which was comparable to 3.2% in the leaf of transgenic rice plants [1]. The GUS activity in the examined leaf and root tissues of transgenic maize plants was more than 500-fold higher than that in the comparable

tissues of control maize plants. We also observed that the level of *gus* expression varied in different tissues and in the same tissues at different developmental stages. Of all tissues tested, vegetative shoot tips and floral meristems showed the highest level of GUS expression. Actively cell-dividing and -growing regions showed a much higher level of expression compared to differentiated tissues. These results were not surprising, because the *Act1* promoter exhibited a similar pattern of expression in rice [28].

Some primary transgenic maize plants presented a *gus* expression pattern different from most transformants in the same transformation events, such as higher GUS activity only in the vascular tissues of roots (Fig. 3c) and in the developing anther primordia (Fig. 3l). The direct cause of those variations is unknown. One possible explanation is the tissue culture-derived abnormalities [14] or greenhouse conditions.

Acknowledgements

We are grateful to Dr. Charles L. Armstrong (Monsanto Agriculture Company, St. Louis, MO) for providing the Hi-II embryogenic callus. The construction of plasmids was performed at Cornell University. The development of transformation system, the transformation and the analysis of transformants were performed at Michigan State University.

References

- [1] W. Zhang, D. McElroy and R. Wu, Analysis of rice *Act1* 5' region activity in transgenic rice plants. *Plant Cell*, 3 (1991) 1155–1165.
- [2] A. Jahne, D. Becker, R. Brettschneider and H. Lorz, Regeneration of transgenic, microspore-derived, fertile barley. *Theor. Appl. Genet.*, 89 (1994) 525–533.
- [3] D. Becker, R. Brettschneider and H. Lorz, Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. *Plant J.*, 5 (1994) 299–307.
- [4] N.S. Nehra, R.N. Chibbar, N. Leung, K. Caswell, C. Mallard, L. Steinhauer, M. Baga and K.K. Kartha, Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. *Plant J.*, 5 (1994) 285–297.
- [5] P. Barcelo, C. Hagel, D. Becker, A. Martin and H. Lorz, Transgenic cereal (tritordeum) plants obtained at high efficiency by microprojectile bombardment of inflorescence tissue. *Plant J.*, 5 (1994) 583–592.
- [6] D. McElroy, W. Zhang, J. Cao and R. Wu, Isolation of an efficient actin promoter for use in rice transformation. *Plant Cell*, 2 (1990) 163–171.
- [7] D. McElroy, A.D. Blowers, B. Jenes and R. Wu, Construction of expression vectors based on the rice actin 1 (*Act1*) 5' region for use in monocot transformation. *Mol. Gen. Genet.*, 231 (1991) 150–160.
- [8] K. Schledzewski and R.R. Mendel, Quantitative transient gene expression: comparison of the promoters for maize polyubiquitin 1, rice actin 1, maize-derived *Emu* and CaMV 35S in cells of barley, maize and tobacco. *Transgen. Res.*, 3 (1994) 249–255.
- [9] H. Zhong, M.G. Bolyard, C. Srinivasan and M.B. Sticklen, Transgenic plants of turfgrass (*Agrostis palustris* Huds.) from microprojectile bombardment of embryogenic callus. *Plant Cell Rep.*, 13 (1993) 1–6.
- [10] H. Zhong, B. Sun, D. Warkentin, S. Zhang, R. Wu, T. Wu and M.B. Sticklen, The competence of maize shoot meristems for integrative transformation and inherited expression of transgenes. *Plant Physiol.*, 110 (1996) In press.
- [11] T. Murashige and F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15 (1962) 473–497.
- [12] H. Zhong, C. Srinivasan and M.B. Sticklen, In-vitro morphogenesis of corn (*Zea mays* L.) I. Differentiation of multiple shoot clumps and somatic embryos from shoot tips. *Planta*, 187 (1992) 483–489.
- [13] C.L. Armstrong, C.E. Green and R.L. Phillips, Development and availability of germplasm with high type II culture formation response. *Maize Genet. Coop. Newslett.*, 65 (1991) 92–93.
- [14] W.J. Gordon-Kamm, T.M. Spencer, M.L. Mangano, T.R. Adams, R.J. Daines, W.G. Start, J.V. O'Brien, S.A. Chambers, W.R. Adams, N.G. Willetts, T.B. Rice, C.J. Mackey, R.W. Krueger, A.P. Kausch and P.G. Lemaux, Transformation of maize cells and regeneration of fertile transgenic plants. *Plant Cell*, 2 (1990) 603–618.
- [15] D.A. Walters, C.S. Vetsch, D.E. Potts and R.C. Lundquist, Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants. *Plant Mol. Biol.*, 18 (1992) 189–200.
- [16] R.W. Thornburg, G. An, T. Cleveland, R. Johnson and C.A. Ryan, Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyl transferase gene fusion in transgenic tobacco plants. *Proc. Natl. Acad. Sci. USA*, 84 (1987) 744–748.
- [17] C.J. Thompson, N.R. Movva, R. Tizard, R. Cramer, J.E. Davies, M. Lauwereys and J. Botterman, Characterization of the herbicide-resistance gene bar from *Streptomyces hygroscopicus*. *EMBO J.*, 6 (1987) 2519–2523.
- [18] C.C. Chu, C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chy and F.Y. Bi, Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen source. *Sci. Sin. (Peking)*, 18 (1975) 659–668.

- [19] S. Zhang, Z.S. Guo, Y.Q. Qian, Q.G. Cai and Y.L. Zhou, Factors influencing isolation, division and plant regeneration in maize (*Zea mays* L.) protoplast culture. *Chin. J. Bot.*, 2 (1990) 18–25.
- [20] E.M. Southern, Detection of specific sequences among DNA fragments separation by gel electrophoresis. *J. Mol. Biol.*, 98 (1975) 503–517.
- [21] R.A. Jefferson, Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.*, 5 (1987) 387–405.
- [22] W.N. Burnette, 'Western blot': electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.*, 112 (1981) 195.
- [23] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, 227 (1970) 680–685.
- [24] H. Towbin, T. Staehelin and J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc. Natl. Acad. Sci. USA*, 76 (1979) 4350.
- [25] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72 (1976) 248–254.
- [26] M.J. Battra and T.C. Hall, Histochemical analysis of CaMV 35S promoter- β -glucuronidase gene expression in transgenic rice plants. *Plant Mol. Biol.*, 15 (1990) 527–538.
- [27] R. Tereda and K. Shimamoto, Expression of CaMV 35S-GUS gene in transgenic rice plants. *Mol. Gen. Genet.*, 220 (1990) 389–392.
- [28] D. McElroy, M. Rothenberg, K.S. Reece and R. Wu, Characterization of the rice (*Oryza sativa*) actin gene family. *Plant Mol. Biol.*, 15 (1990) 257–268.